Exhibit 1

The 5′ Nuclease Assay

5′ Nuclease Activity

AmpliTaq Gold® DNA Polymerase has 5′ exo-nuclease activity. The **5′ exo-nuclease activity** of AmpliTaq® Polymerase and **FRET** (*Fluorescent Resonant Energy Transfer*) makes it possible to detect PCR amplification in Real-Time. The 5′ exo-nuclease activity of the enzyme acts upon the surface of the template to remove obstacles downstream of the growing amplicon that may interfere with its' generation. The 5′ nuclease assay uses this activity in real time detection.

Figure 9: Taq polymerase activity

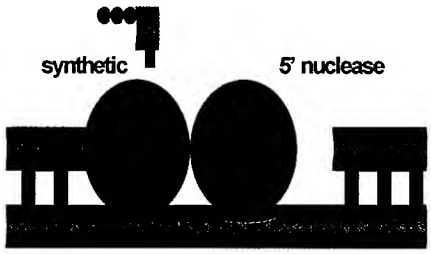
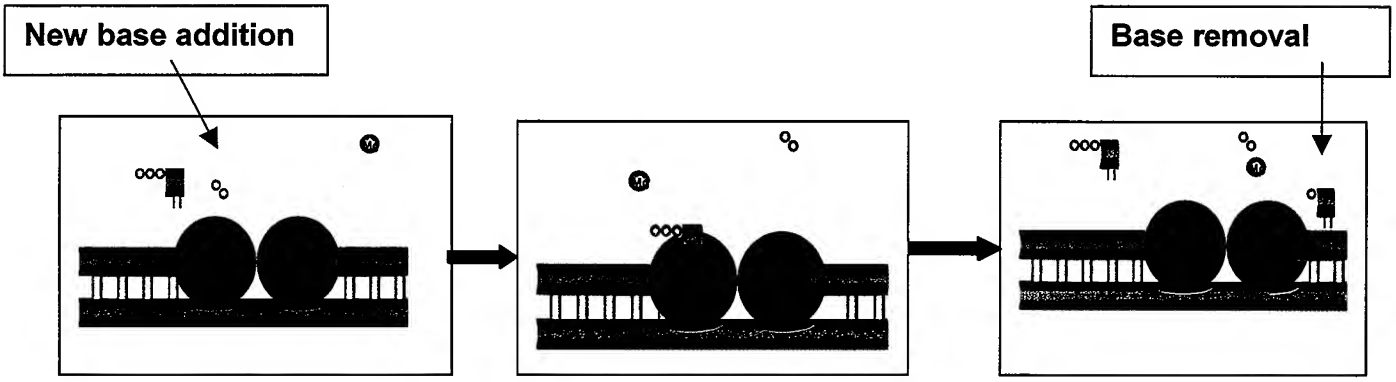


Figure 10: 5′ Exo-Nuclease Activity of Taq Polymerase:

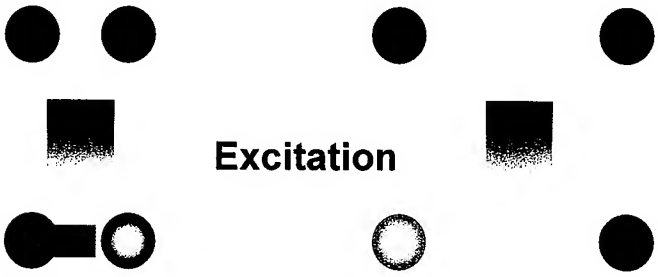
Here the polymerase is adding bases to a growing chain of DNA. Subsequently, the polymerase is removing DNA that is downstream, impeding its' capability to synthesize the new strand.



FRET (Fluorescent Resonance Energy Transfer)

FRET or Florescent Resonance Energy Transfer technology is utilized in the 5′ nuclease assay. The principle is that when a high-energy dye is in close proximity to a low-energy dye, there will be a transfer of energy from high to low, Figure 11.

Figure 11: FRET



The 5′ Nuclease Assay

In the 5′ nuclease assay, an oligonucleotide called a **TaqMan® Probe** is added to the PCR reagent master mix. The probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5′ exo-nuclease activity of the enzyme cleaves the probe, Figure 12 through 14.

Figure 12: The 5′ Nuclease Assay

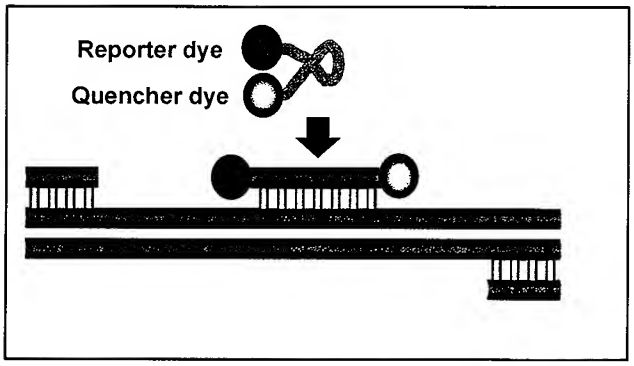
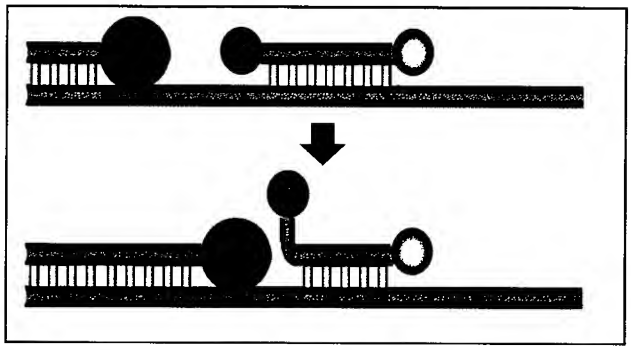


Figure 13: Polymerase collides with TaqMan® Probe



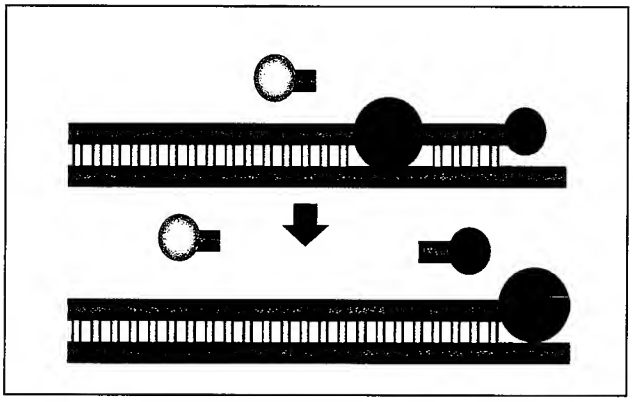


Figure 14: Cleavage of the TaqMan® Probe

The **TaqMan® Probe** is designed with a high-energy dye termed a **Reporter** at the 5′ end, and a low-energy molecule termed a **Quencher** at the 3′ end. When this probe is intact and excited by a light source, the Reporter dye's emission is suppressed by the Quencher dye as a result of the close proximity of the dyes, Figure 15.

When the probe is cleaved by the 5′ nuclease activity of the enzyme, the distance between the Reporter and the Quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease.

Figure 15: Increased florescence activity due to the cleaved probe

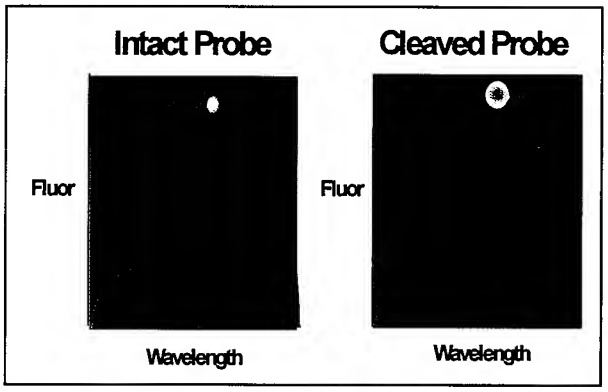


Exhibit 2

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Simultaneous Detection of *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus pneumoniae* in Suspected Cases of Meningitis and Septicemia Using Real-Time PCR

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A single-tube 5′ nuclease multiplex PCR assay was developed on the ABI 7700 Sequence Detection System (TaqMan) for the detection of *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus pneumoniae* from clinical samples of cerebrospinal fluid (CSF), plasma, serum, and whole blood. Capsular transport (*ctrA*), capsulation (*bexA*), and pneumolysin (*ply*) gene targets specific for *N. meningitidis, H. influenzae,* and *S. pneumoniae,* respectively, were selected. Using sequence-specific fluorescent-dye-labeled probes and continuous real-time monitoring, accumulation of amplified product was measured. Sensitivity was assessed using clinical samples (CSF, serum, plasma, and whole blood) from culture-confirmed cases for the three organisms. The respective sensitivities (as percentages) for *N. meningitidis, H. influenzae,* and *S. pneumoniae* were 88.4, 100, and 91.8. The primer sets were 100% specific for the selected culture isolates. The *ctrA* primers amplified meningococcal serogroups A, B, C, 29E, W135, X, Y, and Z; the *ply* primers amplified pneumococcal serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10A, 11A, 12, 14, 15B, 17F, 18C, 19, 20, 22, 23, 24, 31, and 33; and the *bexA* primers amplified *H. influenzae* types b and c. Coamplification of two target genes without a loss of sensitivity was demonstrated. The multiplex assay was then used to test a large number (n = 4,113) of culture-negative samples for the three pathogens. Cases of meningococcal, *H. influenzae,* and pneumococcal disease that had not previously been confirmed by culture were identified with this assay. The *ctrA* primer set used in the multiplex PCR was found to be more sensitive ($P < 0.0001$) than the *ctrA* primers that had been used for meningococcal PCR testing at that time.

Bacterial meningitis is a serious and sometimes fatal infection affecting the central nervous system. Traditional laboratory diagnostic methods of culture for the identification of bacterial meningitis pathogens take up to 36 h or more. Furthermore, it has been observed that following an increase in the practice of starting antimicrobial therapy prior to clinical sample collection, the ability to confirm the pathogenic microorganisms of bacterial meningitis and septicemia has decreased by approximately 30% (8). This has been noted in Public Health Laboratory Service (PHLS) Communicable Disease Surveillance Centre (CDSC) data which show a growing discrepancy between the numbers of clinically suspected and culture-confirmed cases of bacterial meningitis and septicemia, with particular reference to meningococcal infection in England and Wales (17). To address this problem, nonculture methods like PCR have been employed (14) and shown to confirm additional cases of meningococcal disease (18).

The identification of all bacterial pathogens would be desirable, and to this end, amplification of conserved ribosomal nucleotide sequences has provided a strategy for universal detection of bacteria. Unfortunately, this approach is compromised by the presence of residual bacterial DNA contaminating the manufacture of commercially available reagents (4), which has frustrated attempts to exploit the 16S rRNA gene to develop a highly sensitive PCR assay for the universal detection of bacterial causes of meningitis (7).

The 7700 Sequence Detection System (ABI, Warrington, United Kingdom), known as TaqMan, enables amplification and detection to be carried out at the same time in a closed-tube system. Continuous real-time PCR monitoring permits the rapid throughput of large numbers of specimens in a highly standardized format. Multiplex PCR is particularly economical for small-volume samples such as cerebrospinal fluid (CSF) and pediatric blood specimens (10), which constitute a high proportion of samples referred to the PHLS Meningococcal Reference Unit (MRU) for testing. Furthermore, the closed-tube format reduces the chances of contamination. Recent developments in this laboratory have exploited the TaqMan platform to enable the introduction of sensitive and specific assays for the nonculture detection of *Neisseria meningitidis* (14). Advances in real-time PCR technology have now made possible the selective amplification of multiple genes in one reaction vessel by utilizing spectrally distinct phosphoramidite dye-labeled probes. Thus, multiple targets can be specifically identified in a single assay, obviating the need for repeated analysis.

The three major pathogens causing bacterial meningitis are *N. meningitidis*, *Streptococcus pneumoniae,* and *Haemophilus influenzae* type b (Hib); these three organisms accounted for 88.9% of all bacterial meningitis in England and Wales re-

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TABLE 1. Sequences and position of oligonucleotide primers and probes

Gene target	Sequence (5′ to 3′)		
	Forward primer	Reverse primer	Dye-labeled probe[a]
ctrA	617-GCTGCGGTAGGTGGTTCAA-635	727-TTGTCGCGGATTTGCAACTA-708	6-FAM-680-CATTGCCACGTGTCAGCTGCACAT-657
bexA	142-GGCGAAATGGTGCTGGTAA-160	241-GGCCAAGAGATACTCATAGAACGTT-217	TET-189-CACCACTCATCAAACGAATGAGCGTGG-163
ply	894-TGCAGAGCGTCCTTTGGTCTAT-915	974-CTCTTACTCGTGGTTTCCAACTTGA-950	VIC-941-TGGCGCCCATAAGCAACACTCGAA-918

[a] 6-FAM, 6-carboxyfluorescein; TET, tetrachloro-6-carboxyfluorescein.

ported to the PHLS CDSC in 1998 (M. Ramsay, personal communication).

Prior to the introduction of Hib conjugate vaccination, more than 95% of invasive *H. influenzae* disease was caused by Hib (30). In countries where vaccination has been implemented, the incidence of invasive Hib disease has decreased by upwards of 90% (1). Despite the success of the Hib vaccination program that began in October 1992, laboratory reports of Hib disease continue to occur in England and Wales, with 20 bacteremia reports and 26 cases of meningitis and/or encephalitis in 1997 (PHLS CDSC). Furthermore, the reemergence of invasive Hib disease in a well-vaccinated population has been noted (13), emphasizing the necessity for continuous postvaccine surveillance.

N. meningitidis is a cause of meningitis and septicemia in adults and children and is now the major cause of bacterial meningitis in England and Wales, causing 71.9% of bacterial meningitis cases in 1998. These were predominantly caused by serogroups B and C. The annual incidence of meningococcal disease varies between 1 and 4 per 100,000 of the population, although recently the United Kingdom has experienced relatively high disease rates of 3 to 5 per 100,000 (25).

S. pneumoniae is the major cause of childhood invasive bacterial disease where Hib disease has been eliminated by vaccination (26) and is the second most frequently reported cause of septic meningitis (20, 24). In England and Wales the number of pneumococcal septicemia and meningitis cases reported annually increased substantially between 1982 and 1992 (2). Pneumococci were responsible for 19.1% of meningitis and/or encephalitis cases and for 9.6% of laboratory reports of bacteremia in England and Wales in 1997 (PHLS CDSC).

The developments in meningococcal and pneumococcal polysaccharide-protein conjugate vaccines have spurred the need for accurate laboratory confirmation of these infections in order to monitor the effect of vaccine implementation and continuing effectiveness.

This study outlines the development and evaluation of a single-tube multiplex real-time PCR for the simultaneous detection of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* in clinical samples using the TaqMan system. The sensitivity and specificity for the detection of the three major meningitis-causing pathogens are assessed. The application of the multiplex PCR as an epidemiological tool for improved nonculture diagnosis and case ascertainment with a large collection (4,113) of culture-negative specimens referred for meningococcal PCR testing is presented.

MATERIALS AND METHODS

Bacterial strains and culture methods. (i) Sensitivity. The sensitivity of each of the primer sets was evaluated using samples from culture-confirmed cases of meningococcal disease (157 plasma, 36 CSF, 31 serum, 31 whole-blood–EDTA, and 8 throat swab samples), *H. influenzae* disease (6 CSF samples, 2 plasma samples, and 1 whole-blood–heparin sample), and pneumococcal disease (23 CSF samples and 13 serum or plasma samples) that had been obtained from diverse sources. For determination of comparative sensitivities of the primer sets in multiplex and individual PCR formats, serial dilutions (undiluted to 10^{-4}) of quantitated DNA preparations for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* were tested. Different concentrations of template DNA from each organism were tested in combination in order to ascertain the ability of the assay to coamplify multiple gene targets.

(ii) Specificity. The specificities of the three primer sets were determined using genomic DNAs from bacteria and viruses most likely to be present in CSF and blood samples and from other *Neisseria* species. The bacterial strains obtained from the Clinical Microbiology Laboratory at Manchester Public Health Laboratory had been isolated from blood or CSF samples and stored in Microbank vials (Pro-lab Diagnostics, Neston, Wirral, United Kingdom) at −80°C. Additional *S. pneumoniae* strains were obtained from the PHLS National Collection of Type Cultures (NCTC 11887, NCTC 11899, NCTC 11903, NCTC 11904, and NCTC 11908). These strains were cultured overnight on 5% (vol/vol) blood agar (Oxoid, Basingstoke, United Kingdom). The *H. influenzae* strains were obtained from the National Collection of Type Cultures (NCTC 8466, NCTC 8467, NCTC 8469, NCTC 8470, NCTC 8455, and NCTC 8473) and cultured on heated blood agar (Oxoid) at 37°C in 5% CO_2. Staphylococcal species, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were cultured under aerobic conditions. In addition, nine *Escherichia coli* isolates, six *Staphylococcus aureus* isolates, two *Enterobacter cloacae* isolates, one *Streptococcus mitis* isolate, and one group B streptococcus isolate from culture-confirmed whole-blood samples were tested. Herpes simplex virus and varicella-zoster virus isolated from tissue culture, cytomegalovirus from PCR-positive blood samples, and hepatitis B virus from antigen-positive serum samples were also tested in the system. Additionally, extracted human genomic DNAs from 46 healthy adults were tested.

Quantitation of DNA in bacterial cultures. A sweep of colonies from a pure culture obtained using a sterile cotton swab was emulsified in 2 ml of sterile injectable water in a microbiological class 2 safety cabinet. Using a spectrophotometer (Pharmacia, St. Albans, England) set at 650 nm, the bacterial suspension was standardized to an optical density of 0.1 and adjusted to a concentration of approximately 20,000 bacteria/ml, which represents 40 bacteria per 2 μl of inoculum.

DNA extraction. For the clinical isolates and samples, a 100-μl aliquot of standardized suspension or sample was added to 1 ml of DNAzol (Life Technologies, Paisley, Scotland), vortexed, and incubated for 5 min at 20°C. A 500-μl volume of 100% ethanol was added, and the tube was vortexed and incubated for a further 10 min at 20°C. Following centrifugation at 12,000 × *g* for 10 min, the supernatant was aspirated and a further 1 ml of 75% (vol/vol) ethanol was added to the tube, vortexed, and centrifuged at 12,000 × *g* for 5 min. The supernatant was aspirated (with care being taken to remove any residual ethanol), resuspended in 50 μl of sterile water added to the tube, and incubated for a minimum of 10 min in a Dri-bath (Barnstead, Dubuque, Iowa) at 50°C.

PCR design. Oligonucleotide primers and dye-labeled probes (Table 1) were designed using the ABI Primer Express Software Package based on previously published *ctrA* (12), *bexA* (19), and *ply* (32) gene sequences. The *ctrA* sequence-specific probe was 6-carboxyfluorescein labeled, the *bexA* probe was tetrachloro-6-carboxyfluorescein labeled, and the *ply* probe was VIC (chemical name not disclosed by ABI at present) labeled.

PCR components and amplification profile. Based on a 25-μl reaction volume, the master mixture was prepared from the TaqMan Universal Master Mix kit (ABI). Briefly, this comprises a 300 nM concentration of each oligonucleotide primer; 25 nM 6-carboxyfluorescein-labeled probe; 100 nM (each) VIC and tetrachloro-6-carboxyfluorescein fluorescently labeled probes; 5.5 mM $MgCl_2$; 200 μM (each) deoxynucleoside triphosphates dATP, dCTP, dGTP, and dUTP; and 0.125 U of *Taq* DNA polymerase. A negative (no-template) control and

TABLE 2. Sensitivity of meningococcal PCR with culture-confirmed cases of meningococcal disease

Sample type	*N. meningitidis ctrA* result		Sensitivity (%)
	No. positive	No. negative	
CSF	32	4	88.9
Plasma	142	15	90.4
Serum	25	6	80.6
Whole blood-EDTA	28	6	82.4
Throat swab	8	0	100
Other[a]	2	0	100
Total	237	31	88.4

[a] Samples defined as fluid and aspirate.

control DNA preparations (2 μl) for each of the bacterial pathogens were included in every run. DNA was amplified with the TaqMan system using the following cycling parameters: heating at 95°C for 10 min followed by 45 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min. Real-time PCR results were based on the fluorescence readings taken by the TaqMan machine, which are used to calculate a baseline reading for each reaction. The cycle threshold (C_T) value is the PCR cycle number (out of 45) at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence. If no increase in fluorescent signal is observed after 45 cycles, the sample is assumed to be negative.

Clinical evaluation of culture-negative samples. After establishing the specificity and sensitivity of the multiplex assay, it was used to assess the incidences of the three pathogens in 4,113 samples that had been submitted to the Meningococcal Reference Unit for meningococcal PCR testing. In addition, the sensitivity of the multiplex *ctrA* primers was compared with that of the set reported by Guiver et al. (14) that had been used to initially test the samples. The samples had been submitted between December 1998 and May 1999 and included plasma (n = 2,540), serum (n = 655), CSF (n = 451), whole-blood–EDTA (n = 398), and whole-blood–heparin (n = 9) samples. Miscellaneous specimen types (n = 4) included eye swab, urine, and pus. Patient ages ranged from 0 to 90 years. Clinical findings were suggestive of meningitis and/or septicemia being part of the differential diagnosis. These samples had been DNAzol extracted and stored at −20°C for between 2 and 8 months after initial meningococcal PCR testing. Before PCR screening for the three pathogens in the multiplex format, the samples were vortexed to resuspend any bacterial DNA. Any PCR-positive result was confirmed by testing with single primer sets for each gene target (*ctrA*, *bexA*, and *ply*). In addition, newly identified *N. meningitidis ctrA*-positive samples were tested with the serogrouping *siaD* PCR assay as a means of confirmation (3). Where it was not possible to confirm a multiplex result, the original specimen was reextracted and retested using the single primer in an attempt to confirm the initial result.

Statistical analysis. Differences between the results obtained with the *N. meningitidis ctrA* primer set and with the previously reported set were analyzed for statistical significance using McNemar's test.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported here are as follows: *N. meningitidis ctrA*, M80593; *H. influenzae bexA*, M19995; and *S. pneumoniae ply*, X52474.

RESULTS

Assay evaluation. (i) *N. meningitidis ctrA* PCR. The sensitivity of the meningococcal *ctrA* PCR was 88.4% when tested against samples from culture-confirmed cases of meningococcal disease (Table 2). There was no difference in the sensitivity of the *ctrA* primer set when compared in multiplex and single-primer-set formats using serially diluted *N. meningitidis* DNA (Table 3). The *ctrA* primer set amplified DNAs from meningococcal serogroups A, B, C, 29E, W135, X, Y, and Z and diverse serotypes and sero-subtypes. The primers did not amplify DNA from any of the other bacterial and viral DNA extracts tested (100% specificity) (Table 4). There was no cross-reaction with human genomic DNA.

TABLE 3. Comparative end point sensitivities of primer sets in multiplex and single-set formats

Target	Assay format	C_T value[a] at the following dilution of DNA:				
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}
N. meningitidis ctrA	Multiplex	26.94	30.39	32.91	37.25	>45.0
	Single	26.85	30.07	34.25	38.73	>45.0
H. influenzae bexA	Multiplex	28.54	31.58	34.62	>45.0	>45.0
	Single	28.49	32.07	37.96	>45.0	>45.0
S. pneumoniae ply	Multiplex	29.41	32.26	35.40	36.32	>45.0
	Single	29.74	32.06	35.28	36.50	>45.0

[a] The C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence; a value of >45.0 is deemed to be negative.

(ii) *H. influenzae bexA* PCR. The sensitivity of the *bexA* PCR when tested against nine samples from culture-confirmed cases of Hib disease was 100%.There was no difference in the sensitivity of the *bexA* primer sets when compared in multiplex and single-primer-set formats using serially diluted *H. influenzae* DNA (Table 3). The *bexA* primer set amplified DNAs from *H. influenzae* Pittman types b and c. The primers did not amplify Pittman type a, d, e, or f. There was no cross-reactivity with any of the other bacterial and viral DNA extracts tested (100% specificity) (Table 4) and no cross-reaction with human genomic DNA.

TABLE 4. Specificities of *N. meningitidis ctrA*, *H. influenzae bexA*, and *S. pneumoniae ply* primers in multiplex and single PCR formats

Organism(s)	No. tested	No. (%) reactive with the following primer set:		
		ctrA	*bexA*	*ply*
N. meningitidis serogroup A	3	3 (100)		
N. meningitidis serogroup B	24	24 (100)		
N. meningitidis serogroup C	25	25 (100)		
N. meningitidis serogroup X	3	3 (100)		
N. meningitidis serogroup Y	3	3 (100)		
N. meningitidis serogroup Z	3	3 (100)		
N. meningitidis serogroup 29E	3	3 (100)		
N. meningitidis serogroup W135	3	3 (100)		
N. meningitidis, not groupable	5	5 (100)		
H. influenzae Pittman type b	9		9 (100)	
H. influenzae Pittman type c	1		1 (100)	
S. pneumoniae types 1, 2, 3, 4, 5, 6, 7, 8, 9, 10A, 11A, 12, 14, 15B, 17F, 18, 19, 20, 22, 23, 24, 31, and 33	37			37 (100)
Other[a]	89			
Total	253	72 (100)	10 (100)	37 (100)

[a] Other bacterial strains tested were *Neisseria lactamica* (n = 5), *N. gonorrhoeae* (n = 4), *N. sicca* (n = 1), *N. flavescens* (n = 1), *N. cinerea* (n = 1), *N. elongata* (n = 1), *N. pharyngis* (n = 1), *N. polysaccharidae* (n = 1), *Enterococcus faecalis* (n = 5), Lancefield group B streptococci (n = 5), coagulase-negative staphylococci (n = 5), methicillin-resistant *S. aureus* (n = 5), *E. coli* (n = 5), *E. coli* K1 (n = 1), *P. aeruginosa* (n = 5), diphtheroids (n = 5), *Proteus mirabilis* (n = 5), *Acinetobacter* sp. (n = 5), *K. pneumoniae* (n = 5), *Moraxella catarrhalis* (n = 5), *E. cloacae* (n = 5), and *H. influenzae* types a (n = 1), d (n = 1), e (n = 1), and f (n = 1). Viruses tested were herpes simplex virus types 1 and 2 (n = 4), varicella-zoster virus (n = 1), cytomegalovirus (n = 2), and hepatitis B virus (n = 2).

TABLE 5. Coamplification of gene targets

Template	C_T value[a] for the following target gene:		
	ctrA	*bexA*	*ply*
N. meningitidis	38.00		
H. influenzae		31.37	
S. pneumoniae			26.40
N. meningitidis plus *H. influenzae*	39.44	31.69	
N. meningitidis plus *S. pneumoniae*	36.29		27.07
H. influenzae plus *S. pneumoniae*		31.33	26.95

[a] C_T value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence.

(iii) *S. pneumoniae ply* PCR. The *S. pneumoniae ply* PCR sensitivity was assessed using 36 samples from culture-confirmed cases of *S. pneumoniae* disease and was determined to be 91.3% for CSF and 92.3% for serum or plasma, giving an overall sensitivity of 91.8%. There was no difference in the sensitivity of the *ply* primer set when compared in multiplex and single-primer-set formats using serially diluted *S. pneumoniae* DNA (Table 3). The primer set amplified the 23 pneumococcal serotypes tested (serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10A, 11A, 12, 14, 15B, 17F, 18C, 19, 20, 22, 23, 24, 31, and 33). There was no cross-reactivity with any of the other bacterial and viral DNA extracts tested (100% specificity) (Table 4) and no cross-reaction with human genomic DNA.

Multiple target amplification. Two different gene targets were tested using culture extracts and were shown to coamplify without a loss in sensitivity (Table 5). Three samples from which one organism had been cultured and one sample previously PCR positive were simultaneously positive with two of the three gene targets. These results were reproducible with the respective individual PCR assay. Of the three samples PCR positive for both *N. meningitidis* and *S. pneumoniae*, two had been confirmed as *N. meningitidis* infections by culture. Hib was cultured from the *H. influenzae* and *S. pneumoniae* PCR-positive sample (Table 6). These results were reproducible upon reextraction of the original sample.

Culture-negative samples. By testing a large number of culture-negative samples, the *N. meningitidis* primers developed for use in the multiplex assay were found to be significantly more sensitive ($P < 0.0001$) than the previously reported *ctrA* primer set (14). This represented an improvement in the meningococcal detection rate of 2.9% (13.0 versus 15.9% of total *ctrA* PCR-positive samples), or 87 additional cases identified by PCR alone. Of these samples, 53.8% were confirmed as serogroup B or C by *siaD* PCR. Conversely, 62 previously *ctrA* PCR-positive results were not detected using the multiplex *ctrA* primer set on repeat testing. Of these, 24.2% had been confirmed by *siaD* PCR. The total number of specimens *ply* PCR reactive by the multiplex assay was 73, of which 48 (65.7%) were confirmed using the *ply* primer set only. This represents an additional 46 cases confirmed by PCR alone, as two patients were *ply* positive for CSF and plasma samples. One sample not previously Hib culture positive was identified with the *bexA* primers in the multiplex assay (data from the PHLS CDSC).

In total, 49 samples of 736 (6.6%) which were positive by the multiplex assay were not confirmed by the appropriate single-primer-set PCR, of which 46 (93.9%) had a C_T value of greater than 34 of 45 PCR cycles. Twenty-five PCR screen-positive samples could not be tested further due to insufficient specimen amount.

DISCUSSION

Due to the problems associated with the development of a sensitive and specific universal PCR assay (7), a single-tube multiplex PCR was developed based on *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*, which are responsible for upwards of 80% of cases of bacterial meningitis in developed and developing countries (11).

The *ctrA* gene is unique to *N. meningitidis*, and parts of the gene are highly conserved and common to all meningococcal serogroups (12). A previous study has demonstrated the rapid PCR amplification of the *ctrA* gene by continuous monitoring on the TaqMan system using samples from normally sterile sites (14). The limitation of this assay was the design of the forward primer near the 5′ end of the gene, which contains sequence variation between different meningococcal serogroups, particularly those that contain sialic acid (B, C, Y, and W135) and those that do not (12). The *ctrA* primer set reported here amplified sialic acid-containing and non-sialic acid-containing meningococcal serogroups and was found to be significantly more sensitive than the previously described meningococcal *ctrA* PCR (14). Of the discrepant results between the two *ctrA* assays, a higher proportion of the samples positive with the new primer set were confirmed by *siaD* PCR (53.8%) than were confirmed with the previous *ctrA* primer and probe set (24.2%). The high proportion being confirmed enhances the degree of confidence in the results obtained with the new *ctrA* primer set. Increased sensitivity is most likely due to improved primer design, a characteristic that has been noted by Guiver et al. (14). In addition, amplification of all meningococcal sero-

TABLE 6. Comparison of suspected dual infections

Multiplex PCR result	Sample no.	C_T value[a] for the following gene target:			PCR and/or culture result
		ctrA	*bexA*	*ply*	
N. meningitidis plus *S. pneumoniae*	1	34.36	>45.0	36.39	*N. meningitidis siaD* serogroup C by PCR
	2	36.06	>45.0	41.85	*N. meningitidis* serogroup B by culture
	3	35.09	>45.0	38.81	*N. meningitidis* serogroup C by culture and PCR
S. pneumoniae plus *H. influenzae*	1	>45.0	31.33	39.90	Hib by culture

[a] A value of >45.0 is deemed to be negative.

groups may account for some of the additional positive samples; for example, a case of serogroup A disease not detected with the previous primer set was identified using the new set. The new *ctrA* primers failed to amplify DNA from some samples that had been previously positive; however, almost all of these samples had been weak positives in earlier analyses. These samples remained negative upon repeat testing with the original *ctrA* primer set and also after the sample was reextracted and retested. This failure to repeatedly detect DNA in specimens indicates that degradation of DNA is likely to be occurring and is likely to be caused by long-term storage and/or repeated freezing and thawing of samples. In addition, sampling error is more likely to be associated with previously low-level-positive samples. The gene target is present in low numbers as indicated by the high C_T value, and sample variation would lead to nonreproducible amplification.

The *bexA* gene encodes the capsulation-associated BexA protein present in all capsulated *H. influenzae* strains. These strains express one of six capsular polysaccharides (types a to f) (22). The amplification of the *bexA* gene for the detection of *H. influenzae* in CSF samples has previously been reported, and the gene was shown to be amplified in all six *H. influenzae* types. Van Ketel et al. used a relatively insensitive, gel-based detection system and experienced problems with contamination (31). Optimal primer and probe sets for use in the TaqMan system were developed using the criteria in the Primer Express software (ABI) from the available Hib sequence. This primer and probe set amplified types b and c only, and the inability to detect other serotypes was assumed to be because of nucleotide sequence variation.

The *S. pneumoniae* pneumolysin gene encodes the hemolysin species-specific protein toxin produced intracellularly by all clinically relevant pneumococcal serotypes (21). PCR amplification from clinical material is indicative of invasive pneumococcal infection. There have been several reports of pneumococcal PCR utilizing amplification of the pneumolysin gene (16, 27, 29), with a report of PCR for the detection of *S. pneumoniae* DNA in culture-negative samples where meningitis was the diagnosis (5). This assay utilized the autolysin gene and was evaluated using only a small number of culture-negative clinical samples ($n = 11$).

The pneumococcal PCR developed here was specific for the 23 pneumococcal serotypes tested while simultaneously offering a high level of sensitivity. The 4,113 samples tested were from patients clinically suspected as having meningococcal disease; 48 samples from 46 cases were confirmed as pneumococcal PCR positive. These had not been identified by laboratory culture, emphasizing the beneficial impact of including pneumococcal PCR in the routine diagnostic testing strategy.

Data suggest that 1% of all cases of meningitis are due to more than one pathogen (9), and such cases have recently been reported in the literature (6, 23). However, traditional laboratory methods may not always identify multiple pathogens in a single clinical sample, as identification from culture is based on the predominating organism and may be influenced by the use of selective culture media. PCR assays have been shown to amplify multiple pathogens (15, 28), but these assays relied on a nested PCR approach for improved sensitivity. The multiplex PCR in this study coamplified gene targets in a single-round PCR with a correlation between the organism identified by laboratory culture and the one with the lowest C_T value (Table 6). In all cases, the cultured organism has the lower C_T value, suggesting that this was the predominant organism in the specimen. The possibility of cross contamination of extracts was ruled out by reproducing the original results using another extract of the sample. The evidence confirms other observations that on some occasions, more than one organism may be present in a clinical sample and these may be underdetected by traditional laboratory methods.

In cases where it was impossible to confirm the multiplex PCR-positive results, the cycle number to reach the baseline threshold (C_T) value was greater than 34 in 93.9% of specimens. Plasmid titration experiments for the generation of a standard curve have demonstrated that the detection of samples around cycle 35 represents a target input of fewer than 10 copies (14). This is therefore approaching the limits of detection of the PCR, and it would be expected that a positive result would not always be obtained on repeat testing due to sampling error.

By utilizing the available TaqMan technology, the introduction of a three-in-one multiplex PCR enables rapid identification and a high throughput of samples (130 min for 96 specimens), with a modest additional cost for primers and probes in each reaction. The multiplex PCR demonstrated that testing a large number of previously culture-negative specimens provides information on the incidence of meningococcal, *H. influenzae*, and pneumococcal infections in clinical specimens originally referred for meningococcal PCR testing. The potential for guiding clinicians towards the most appropriate antimicrobial therapy and patient management is improved. The inclusion of the multiplex PCR in the routine molecular diagnostic screening regimen would provide a rapid and robust assay for the improved nonculture diagnosis and case ascertainment of meningitis and septicemia.

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